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ASSESSMENT OF A RAPID ENZYME IMMUNOASSAY STICK TEST FOR THE DETECTION OF CIGUATOXIN AND RELATED POLYETHER TOXINS IN FISH TISSUES

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Abstract

We have developed a rapid and simple enzyme immunoassay stick test for ciguatoxin and related polyether toxins in fish tissues. This assay can be used to examine clinically implicated fishes from ciguatera poisonings, corresponding catches of fishes obtained in the same vicinity as those clinically implicated, non-toxic consumed fishes, and nearshore reef fishes. With this assay, 14 clinically documented fishes gave a mean and standard deviation ($\bar{x} \pm$ S.D.) of 3.1 ± 0.7. The corresponding catches of fishes from areas implicated in ciguatera poisonings showed the following percentages of toxicity for *Ctenochaetus strigosus:* Hawaii Island, 45.0%; Kauai, 80.6%; and Oahu, 42.5%. Sixty non-toxic consumed fishes gave a $\bar{x} \pm$ S.D. value of 1.2 ± 0.5, and *Thunnus thynnus,* a pelagic fish never implicated in ciguatera poisoning, gave a $\bar{x} \pm$ S.D. value of 1.1 ± 0.8. All of the clinically implicated fishes were in the borderline to positive value ranges with the stick test. Associated with the high incidence of toxicity of *C. strigosus* from Kauai, *Gambierdiscus toxicus* was found in the gut of all 93 fish samples examined. The stick enzyme immunoassay has proven to be simple, rapid, sensitive, and specific.

Introduction

Ciguatera poisoning, endemic to the tropics and subtropics and a health problem to the consumer, is associated with the consumption of ciguatoxin, a low dalton lipid polyether (Scheuer *et al.,* 1967; Tachibana, 1980; Nukina *et al.,* 1984). The origin of this toxin is the dinoflagellate *Gambierdiscus toxicus* (Yasumoto *et al.,* 1977; Adachi and Fukuyo, 1979). Dinoflagellates also produce other structurally related polyether toxins such as okadaic acid (Tachibana *et al.,* 1981; Murakami *et al.,* 1982) and brevetoxin (Lin *et al.,* 1981; Baden *et al.,* 1981).

Okadaic acid was isolated from the dinoflagellate *Prorocentrum lima* (Murakami *et al.,* 1982) as well as from the sponges in the genus *Halichondria* (Tachibana *et al.,* 1981), and brevetoxin was extracted from the dinoflagellate causing red tide, *Ptychodiscus brevis* (*Gymnodinium breve*) (Lin *et al.,* 1981; Baden *et al.,* 1981). These polyether toxins appear to have an effect on the membranes of nerve and muscle tissues and to induce changes in ion permeability of the cells (Rayner, 1972; Miyahara *et al.,* 1979; Ohizumi *et al.,* 1982).

A major area of study initiated in the past decade has been the development of sensitive and specific assay methods for the assessment of these toxins in contaminated fish tissues. Earlier assays included: (1) the feeding of fish tissues to cats and mongoose (Banner *et al.,* 1961; Bagnis, 1973); and (2) the injection of crude extracts into mice, chicks, and, more recently, mosquitoes (Kosaki *et al.,* 1968; Yasumoto *et al.,* 1971; Kimura *et al.,* 1982; Chungue *et al.,* 1984). These tests lacked both sensitivity and specificity. More recent studies have taken the immunological approach, us-

ing antibodies prepared in sheep and rabbit, following immunization with conjugates of the polyether toxins (Hokama *et al.,* 1977; Kimura *et al.,* 1982; Hokama *et al.,* 1983, 1984, 1985; Baden *et al.,* 1984).

We have developed and assessed a rapid, simple, specific, and sensitive stick test to detect ciguatoxin and related polyether toxins in contaminated fish tissues. The fishes examined included: (1) clinically implicated fishes from ciguatera poisonings; (2) fishes from corresponding catches obtained in the same vicinity with the implicated fishes; (3) portions of non-toxic fishes that were consumed without incident; and (4) fishes from the nearshore waters of Hawaii where toxicity occasionally occurs.

Materials and Methods

Sources of fish samples

The following fishes were obtained through the courtesy of the Department of Health (DOH), State of Hawaii: Clinically implicated fish samples from ciguatera poisonings—1, *Acanthocybium solandri;* 1, *Aprion virescens;* 3, *Caranx* species; 2, *Cephalopholis argus;* 1, *Sphyraena* species; and 1, grouper extract. Corresponding catch fishes associated with the fishes implicated in ciguatera poisonings—93, *Ctenochaetus strigosus;* 16, *Mulloidichthys* species; 6, *Cephalopholis argus;* 3, *Cheilinus rhodochrous;* and 1, *Aphareus furcatus.*

The following fishes were obtained through the courtesy of the Department of Land and Natural Resources (DLNR), State of Hawaii: Non-toxic consumed fishes—43, *Seriola dumerili;* 6, *Elagatis bipinnulatis;* 5, *Sphyraena* species; and 1, *Caranx* species. Reef fishes from the nearshore waters of Oahu and Hawaii Island—*Cheilinus rhodochrous; Kyphosus cinerescens; Ctenochaetus strigosus; Lutjanus kasmira; Acanthurus* species; and *Myripristis* species.

Non-toxic consumed *Thunnus thynnus* was obtained from commercial sources (supermarket).

Source of purified ciguatoxin

Purified ciguatoxin isolated from livers of moray eels (Nukina *et al.,* 1984) was obtained through the courtesy of Professor P. J. Scheuer of the Department of Chemistry, University of Hawaii, Honolulu, Hawaii.

Stick test reagents

The reagents for the stick test were similar to those used in the enzyme immunoassay reported previously (Hokama *et al.,* 1983). These included: (a) methanol fixative, 0.3% hydrogen peroxide (H_2O_2) in absolute methanol, prepared fresh daily; (b) Tris buffer A, 0.05 *M* Tris (hydroxymethyl)aminomethane, pH 7.5 ± 0.05, with 0.1% human serum albumin (HSA) and 0.01% sodium azide; (c) Tris buffer B, prepared as in A, but without HSA and sodium azide; (d) sheep-anti-CTX coupled to horseradish peroxidase (HRP, Type VI, RZ:3:3, Sigma Chemical Co., St. Louis, MO) according to the one-step glutaraldehyde method (Voller *et al.,* 1980), stored in aliquots of 1 ml at −20°C. until ready for use; (e) substrate, 25 ml of 0.3% H_2O_2 in Tris buffer B added to 10 mg of 4-chloro-1-naphthol previously dissolved in 0.125 ml of absolute methanol, mixed thoroughly then filtered through Whatman #1 filter paper, prepared fresh just before use; (f) bamboo sticks (length, 200.0 mm; diameter, 2.5 mm; Mum's Taisei of Hawaii, Honolulu) with the skewer ends coated with Liquid Paper (The

Gillette Co., Rockville, MD). Any excess Liquid Paper was removed in the preparation of the coat, then the coating was allowed to dry thoroughly before use.

Stick test procedure

Fish were sampled by inserting the skewer end of the stick coated with the Liquid Paper into the dorsal-anterior and ventral-posterior sections of one or both sides of the fish. Each stick was inserted 5 times into the flesh at 1 s/insertion. Each fish was examined with six sticks; three in the dorsal-anterior and three in the ventral-posterior portions of the side of the fish. The stick was air dried and then immersed into the fixative for 1 s without shaking. The excess solution was blotted onto tissue paper and the stick was air dried. The stick was then washed in Tris buffer B thoroughly with gentle shaking for 10 s and the excess solution blotted onto tissue paper. The stick was then immersed for 15 to 30 s without agitation into the sheep-anti-CTX-HRP solution previously diluted 1:200 with Tris buffer A just prior to use. The excess antibody conjugate was blotted onto tissue paper. The stick was then washed in two changes of Tris buffer B, 10 s each with gentle shaking. After the excess buffer was blotted, the stick was immersed into 0.3 ml of the substrate. The tube containing the substrate and the stick was shaken, then incubated for 10 min at room temperature. The intensity of the color was compared with a color scheme as follows: 0, essentially no color; 1.0+, slightly bluish-purple; 1.5 to 2.0+, lightly bluish-purple; 2.0 to 2.5+, moderately bluish-purple; 3.0 to 5.0+, moderately to intensely bluish-purple. The results of these reactions were scored as follows: 0 to 2.0+, negative; 2.1 to 2.4+, borderline; and values greater than 2.5+, positive. The fractional values were the result of averaging several sticks per sample.

The reagents for the test were arranged in the following sequence: (a) fixative; (b) Tris buffer B wash 1; (c) sheep-anti-CTX-HRP; (d) Tris buffer B wash 2; (e) Tris buffer B wash 3; and (f) substrate.

Analysis of purified ciguatoxin by the stick test

Purified ciguatoxin in concentrations of 0, 1, 5, and 25 ng/ml in absolute methanol were prepared. The coated sticks were immersed for 2 s in the ciguatoxin-methanol solutions. The sticks were then air dried and examined by the stick test procedure. The fixation step was not performed for the purified toxin. Several sticks were used for each concentration of the toxin solution. The mean and standard deviation of the stick values were obtained for each toxin concentration and plotted as shown in Figure 1.

Examination of the gut contents of Ctenochaetus strigosus

Smears on microslides from the viscera of *C. strigosus* were prepared from each of the 93 specimens obtained from Kauai as part of the corresponding catches. The smears were suspended in 50% glycerol phosphate buffer saline with a cover slip. Each smear was examined at 400× magnification with the phase and light microscope. Twenty fields per smear were examined and the average number of *Gambierdiscus toxicus* per field determined.

All statistical evaluation was determined by the method of Tallarida and Murray (1981).

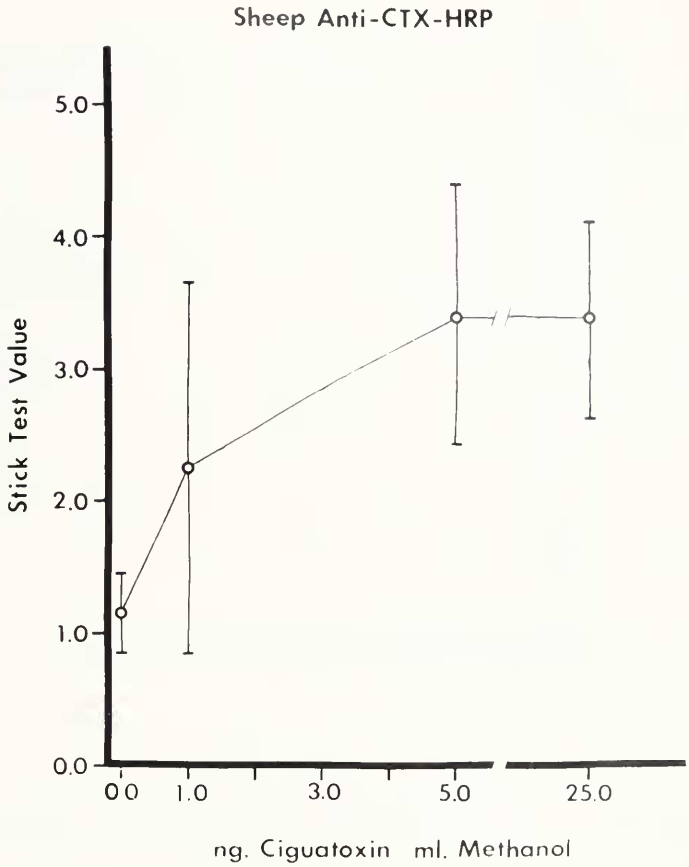

FIGURE 1. Analysis of purified ciguatoxin in methanol by the stick test. The y-axis represents the stick test values and the x-axis the concentration of ciguatoxin. The means (O) and standard deviations (vertical lines) are presented.

RESULTS

Stick test analysis of positive fishes from clinically documented ciguatera poisoning and negative consumed fishes

The assessment of the validity of the stick test in the detection of toxic fishes is summarized in Figure 2. Sixteen clinically implicated raw and cooked (tissues, soup, and gravy) fish samples examined by the stick test gave a $\bar{x} \pm$ S.D. of 3.3 ± 0.7 while 60 non-toxic consumed fishes gave a $\bar{x} \pm$ S.D. of 1.2 ± 0.5 stick test value (Fig. 2). The difference between these two categories was highly significant with $P < 0.005$. Examination of 2 samples of *Thunnus thynnus* gave a stick test value of 1.1 ± 0.8. This value was significantly different from the positive values with $P < 0.01$. *Thunnus thynnus,* a pelagic fish, has not been implicated in ciguatera poisoning. On the basis of this study, the interpretation of the stick test values has been derived and designated as follows: 0–2.0+, negative; 2.1–2.4+, borderline; and values greater than 2.5+ as positive (Fig. 2).

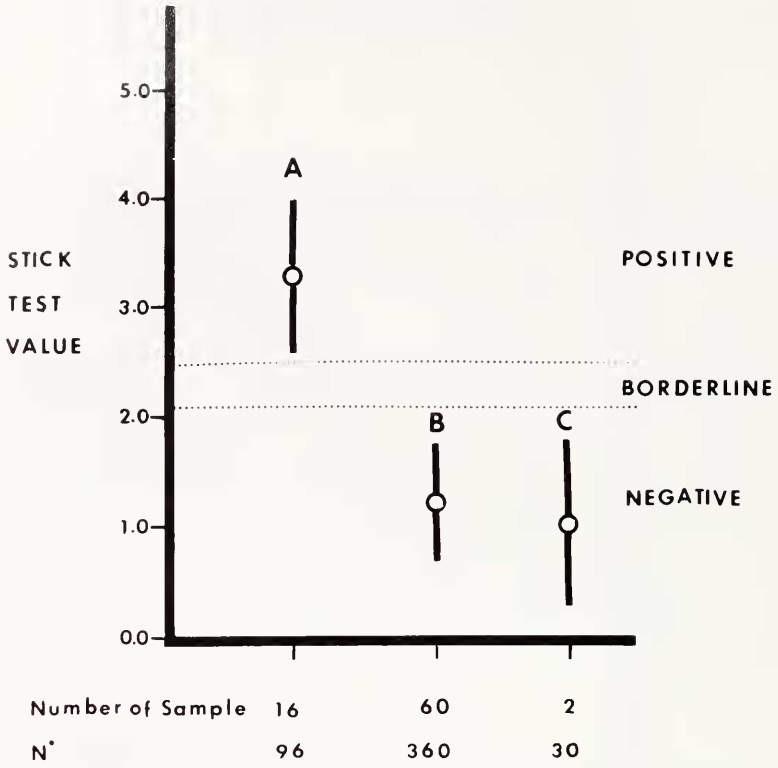

FIGURE 2. Comparison of clinically documented positive fishes associated with ciguatera poisoning (A) and non-toxic consumed negative fishes (B) by the stick test. (C) Represents the *Thunnus thynnus* results. The y-axis represents the stick test values and the x-axis the number of samples. n* = the number of determinations.

Stick test analysis of corresponding catches of fishes and study of the nearshore reef fishes of Hawaii

The results of examining, by the stick test, several species of fishes obtained as corresponding catches with fishes implicated in ciguatera poisonings are shown in Table I. Of the 120 fishes from 4 species examined, 69.2% were considered toxic by the stick test. The positive group also included those fishes in the borderline category. This high percentage is due to *Ctenochaetus strigosus,* which caused a large outbreak of ciguatera poisoning on Kauai in August, 1984 (see Table II). The gut contents of all 93 samples contained 0.1 to 1.5 *Gambierdiscus toxicus* per field at 400× magnification (Fig. 3).

Summary of stick test evaluation of nearshore reef fishes from Oahu, Hawaii Island, and Kauai

These studies are summarized in Tables II, III, and IV. All four species of fishes examined from Oahu came from the Leeward side of the nearshore waters (Table III). Fishes caught in these areas generally have the highest frequency of ciguatera poisoning, in contrast to the Windward side, where essentially no ciguatera poisoning

TABLE I

Examination of several species of fishes from corresponding catches of ciguatera poisoning in Hawaii, 1984

Species	Total number	Stick test	
		Negative	Positive
		No. of fish	
Ctenochaetus strigosus (Kole, surgeon fish)	93	18	75
Cephalopholis argus (Roi, grouper)	6	4	2
Mulloidichthys species (Weke, goat fish)	16	15	1
Cheilinus rhodochrous (Po'ou, wrasse)	3	0	3
Aphareus furcatus (Wahanui, snapper)	1	0	1
Total	120	37	81
%	–	30.8	69.2

has occurred. A high percentage of toxicity is shown by *C. strigosus* (43.5%) and *L. kasmira* (36.5%). Note that the herbivore (*C. strigosus*) gave a higher percentage of toxic samples than either the carnivore (*L. kasmira*) or the two other species examined (*Acanthurus* and *Myripristis*). The positive category includes the borderline samples.

Table IV summarizes the results of the samples of three species from Hawaii Island. A high percentage of positives is found in *C. strigosus* (45.0%) as compared to *K. cinerescens* (0.0%) and *C. rhodochrous* (20.8%).

TABLE II

Comparison of Ctenochaetus strigosus *examined by the stick test from three different islands: Hawaii, Oahu, and Kauai*

Source	Total number	Stick test	
		Negative	Positive
		No. of fish	
Kauai*	93	18	75
	%	19.4	80.6
Hawaii**	51	28	23
	%	55.0	45.0
Oahu***	108	61	47
	%	56.5	43.5

* Samples from corresponding catches of ciguatera poisoning outbreak, August, 1984.

** Samples obtained in areas associated with ciguatera outbreaks from *Cephalopholis argus* and *Cheilinus rhodochrous*, April–August, 1984.

*** Samples from Barbers Point and Ewa, routine monthly survey of Harbor development and control site, 1984.

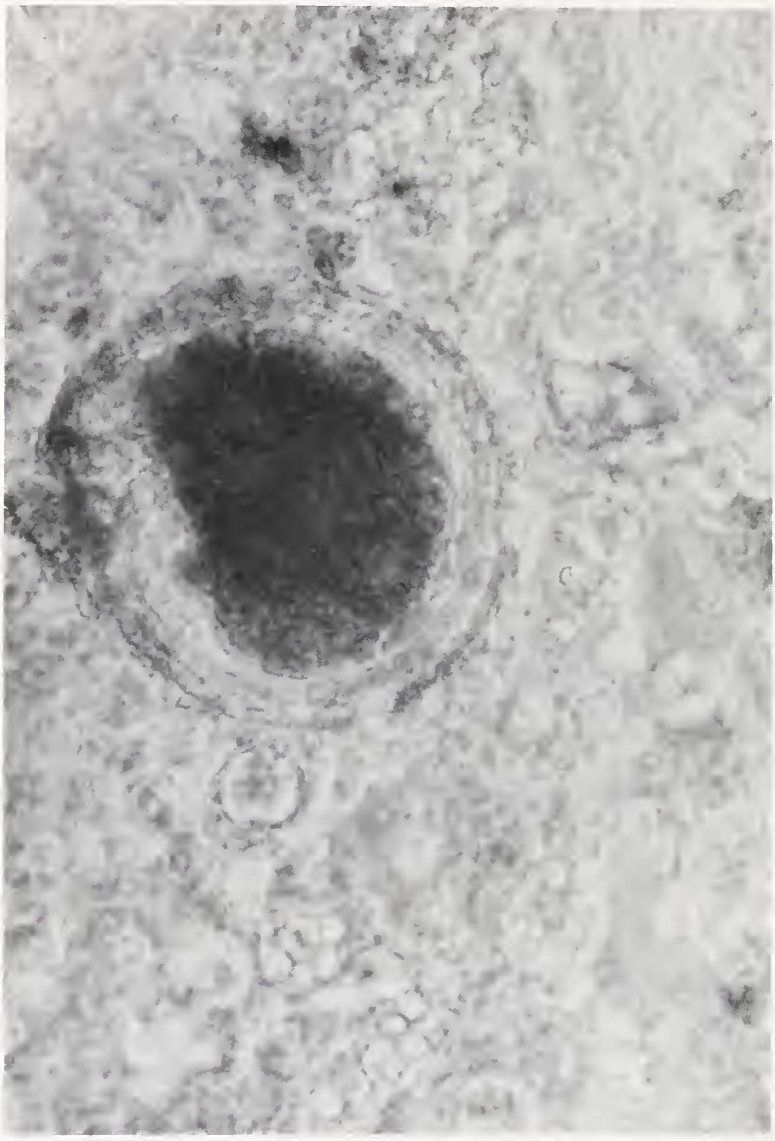

FIGURE 3. A typical photograph of *Gambierdiscus toxicus* from the gut of *Ctenochaetus strigosus* obtained from the nearshore waters of Kauai (400× magnification).

The comparison of *C. strigosus* from the three different islands by the stick test procedure is shown in Table IV. The highest percentage of toxicity was shown in samples from Kauai (80.6%). Samples from Oahu and Hawaii Island were nearly identical, 43.5% and 45.0%, respectively. This is understandable, since the samples from Kauai were part of the corresponding catches of a large ciguatera outbreak due to *C. strigosus*. The Hawaii Island samples of *C. strigosus* were caught on the Leeward side in April of 1984 just prior to small outbreaks of ciguatera poisoning that contin-

TABLE III

Summary of stick test assessment of fishes from Oahu

Species	Total number	Stick test	
		Negative	Positive
		No. of fish	
Ctenochaetus strigosus	108	61	47
(Kole, surgeon fish)	%	56.5	43.5
Lutjanus kasmira	96	61	35
(Taape, blue-line snapper)	%	63.5	36.5
Acanthurus species	16	19	1
(Palani, surgeon fish)	%	76.0	24.0
Myripristis species	12	9	3
(Menpachi, squirrel fish)	%	75.0	25.0
Total	241	149	91
%	–	61.8	38.2

ued throughout 1984. The fishes implicated included *Cephalopholis argus, Ctenochaetus strigosus,* and *Cheilinus rhodochrous.* The Oahu samples of *C. strigosus* were obtained during the autumn of 1984 from the DLNR Barbers Point (Leeward side of Oahu) study. No incidence of ciguatera poisoning was reported during this period (an outbreak occurred in January, 1985, in this area). In part, this may be attributable to the lack of fishing in this area due to the turbid water conditions caused by the deep harbor dredging and construction at Barbers Point.

Microscopic examination of viscera smears from C. strigosus *(Kauai)*

All 93 samples of *C. strigosus* showed *Gambierdiscus toxicus* in their gut contents when smears were examined with the phase microscope at 400× magnification. The range of dinoflagellates/field was 0.1 to 1.5. A typical example of *G. toxicus* is shown in Figure 3.

TABLE IV

Summary of stick test assessment of fishes from Hawaii

Species	Total number	Stick test	
		Negative	Positive
		No. of fish	
Ctenochaetus strigosus	51	28	23
(Kole, surgeon fish)	%	55.0	45.0
Kyphosus cinerescens	28	28	0
(Nenue, rudder fish)	%	100.0	0.0
Cheilinus rhodochrous	24	19	5
(Po'ou, wrasse)	%	79.2	20.8
Total	103	75	28
%	–	72.8	27.2

Discussion

The results of this study show the feasibility of adapting the enzyme immunoassay to a simple and rapid stick test. This procedure retained its sensitivity and specificity using the conventional heterologous sheep-anti-CTX-HRP (Hokama *et al.,* 1977) or the recently prepared monoclonal antibodies to toxic polyethers (Hokama *et al.,* 1985). The success of the procedure is probably due to the selective adsorption or attraction of the lipid toxins in fish tissues by one or more constituents in the Liquid Paper coated onto the bamboo stick. The latter alone without coating had no activity in the assay. Coated sticks alone have shown essentially little activity with values equal to or below the normal value (less than 2.0+). This is attributable in part to the non-specific binding of the antibody enzyme conjugate.

We compared the clinically implicated toxic fishes and the non-toxic consumed fishes with the stick test and demonstrated significant differences in the mean and standard deviation of the stick test values between these two groups with $P < 0.005$. These same samples tested by the enzyme immunoassay (Hokama *et al.,* 1983, 1984, 1985) agreed with the stick test values presented in this report. Hokama *et al.,* (1984) recently showed that the sheep-anti-CTX-HRP detected ciguatoxin and structurally related polyether toxins such as okadaic acid and brevetoxin in a competitive enzyme immunoassay procedure. The stick test also interacted with purified ciguatoxin in a dose-responsive manner (Fig. 1).

Examination of the fishes from corresponding catches showed a high level of toxicity (80.6% positive), especially *Ctenochaetus strigosus* from the island of Kauai during August, 1984, when an outbreak of ciguatera poisoning occurred involving 14 individuals. At least one and up to four *C. strigosus* were consumed by each individual. The viscera of all 93 specimens retrieved from the markets by the DOH contained *Gambierdiscus toxicus.* Outbreaks of ciguatera poisoning occurred in Hawaii during most of 1984, beginning in the early spring months. A moderate to high level of toxicity was also noted in *C. strigosus* (45.0%); with a slightly lower level in *Cheilinus rhodochrous* (21.0%). However, *Kyphosus cinerescens* essentially showed no toxicity though caught in the same area. The toxicity patterns of Oahu appeared similar to that of the island of Hawaii (Tables II and IV).

The radioimmunoassay (Hokama *et al.,* 1977), the enzyme immunoassay (Hokama *et al.,* 1983, 1984), and the stick test (Hokama, 1985) all use the immunological approach for the detection of ciguatoxin and related polyether toxins directly from fish tissues. The stick test differs from the former two tests in its simplicity and rapidity. The stick test also requires no capital equipment, and it can be used at home or in the field. Such a system also may be used for other enzyme immunoassays, perhaps using other coats to selectively adsorb the antigenic or haptenic components onto the stick. Furthermore, the stick test does not require the extraction of tissues, as required for the mouse bioassay (Yasumoto *et al.,* 1971; Kimura *et al.,* 1982) and the recently described mosquito assay (Chungue *et al.,* 1984). However, the stick test is also capable of examining fish tissue extracts. In addition, the stick test can be performed rapidly, while retaining its sensitivity and specificity, for mass screening of fishes on a large commercial level with minimal costs.

In summation, the stick test is simple, rapid, and inexpensive, with sufficient specificity and sensitivity to evaluate the levels of ciguatoxin and related polyether toxins in fishes suspected of potential ciguatera poisoning.

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